CHARACTERIZATION OF EXTRA-AND INTRACELLULAR PHYTASES FROM PROLIVERATING YEAST DURING TEMPEH PRODUCTION

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ABSTRACT

Proliferating yeast species such as *Endomycopsis burtonii, Candida diddensiae* and *Candida tropicalis*, produced both extra- and intracellular phytases during tempeh (soaking of soybean) production. The enzymes were isolated from growth media and the cultured yeast and partially purified by acetone fractionation. Extracellular phytase activities were higher than that of the intracellular enzymes. The extra- and intracellular phytases of *E. burtonii., C. diddensiae* and *C. tropicalis* acted over a narrow pH ranging at 3.2 – 4.8 and the maximum activity at temperatures between 45° – 70°C. Estimated K_m values for extra- and intracellular phytases from yeast were within the range 0.5 – 3.1 x 10^{-4} M. The V_{max} values were within the range 0.004 – 0.19 µmole P_i liberated/min/ml enzyme for the extra- and intracellular phytases of *E. burtonii, C. diddensiae* and *C. tropicalis*. The activation energy for hydrolysis of phytic acid by extra- and intracellular phytases of *E. burtonii, C. diddensiae* and *C. tropicalis* were calculated by the Arrhenius equation being 7,100; 9,100; 13,00, 5,600, 7,100 and 21,700 cal/mole, respectively.

Key words: characterization, Endomycopsis sp., Candida sp., phytase, tempeh.

INTRODUCTION

Tempeh is a traditional Indonesian fermented food made from soaked and cooked soybeans inoculated with a mould, usually of the genus of *Rhizopus*. Soaking of dry soybeans usually forms an integral part of processing method for tempeh production. Eventually, microorganism such as proliferating yeast during soaking of soybeans has been expected to produce either extra- or intra-cellular phytase that are able to reduce phytic acid content of beans as suggested by Jaffe (1981).

Phytase, or myo-inositol hexaphosphate phosphohydrolase (EC 3.1.1.8 or 3.1.3.26) (IUB, 1979), which can hydrolyze phytic acid into myo-inositol and orthophosphate (P_i), has been isolated from several different microorganisms including bacteria (Powar and Jagannathan, 1967), yeast (Nayini and Markakis, 1984; Yanming *et al.*, 1999) and moulds (Sudarmadji, 2000; Sutardi and Buckle, 1988), and also some legume seeds (Sutardi and Buckle, 1986).

The yeast phytase is produced by the most common contaminating microorganisms during tempeh production. The enzyme was isolated, purified and characterized as part of a study to establish whether phytate reduction during tempe production is the result of physical treatments such as soaking, boiling and steaming or due to hydrolysis by phytases produced by yeast contaminating during soaking of raw soybeans overnight.

MATERIALS AND METHODS

Organisms

Yeast that grew well during the soaking of soybeans were isolated and identified as *Endomycopsis burtonii, Candida diddensiae* and *Candida tropicalis*. The yeast cultures were transferred to a Malt Extract Agar (MEA) slant, incubated for 48 h at 30°C and maintained for further experiments.

Cultivation of organisms

Loop of pure culture yeast was inoculated into 10 ml Malt Extract Broth (MEB) in a test tube and incubated for 16 h at 30°C, and then transferred into 100 ml of MEB in a 300 ml erlenmeyer flask, and incubated in an orbital shaking incubator for 24 h at 30°C and 200 rpm. The number of cells in every step of culturing was enumerated by a spread plate method on MEA. Cultivations were carried out in triplicate on at least two different preparations of culture.

Harvesting of organisms

Pure yeast cultures were harvested by centrifugation for 30 min at 4°C and 15,000 x g. Supernatants were collected and used as the source of extracellular phytase, while precipitates were used as the source of intracellular phytase.

Crude enzyme preparation

Extracellular enzyme supernatant obtained from centrifugation of growth media was concentrated by freeze drying (-35°C, 3.75 kPa), thawed, and dialysed for 48 h at 4°C against 0.01 M tris-maleat buffer pH 6.5, then subjected to acetone fractionation.

Intracellular phytase, prepared from the precipitate, was washed with double distilled water and then centrifuged for 15 min at 4°C and 15,000 x g. Precipitate was suspended with 20 ml 2% $CaCl_2$/10 g cells (wet basis) in a 50 ml glass tube, and 10 g of glass beads (0.45 – 0.50 mm diameter) was added. Yeast cells were disrupted in a vortex mixer at maximum speed (Hazen and Cutler, 1982). The disruption periods lasted 20 s each, and were followed by rechilling in an ice bath for 1 min. This was repeated until a total disruption time of 2 min was accumulated (Richards and

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Takai, 1979). The suspension was centrifuged for 30 min at 4°C and 15,000 x g. Precipitate was washed with 10 – 20 ml 2% $CaCl_2$ and disruption was repeated as above and centrifuged for 30 min at 4°C and 15,000 x g. The supernatants were collected and subjected to acetone fractionation. The proportion of cell breakage was determined using method describied by Hazen and Cutler (1982).

Acetone fractionation

Acetone fractionation was carried out as for mould phytase fractionation (Sutardi and Buckle, 1988). The acetone purified enzyme were used for subsequent experiments.

Enzyme assay

Extracellular yeast phytases were assayed in stoppered test-tubes at 70° ± 1°C and 55° ± 1°C for *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively. Intracellular yeast phytases were assayed at 65° ± 1°C, 55° ± 1°C and 45° ± 1°C for *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively, by measuring the rate of increase of P_i using the ascorbic acid method (Watanabe and Olsen, 1965). The reaction mixture had a total volume of 1.20 ml and contained 0.20 ml of 0.1 M acetate buffer, pH 4.2 and 4.8 for extra- and intracellular phytases of *C. diddensiae* and *C. tropicalis*, respectively, except pH 3.9 and 3.2 for the extra- and intracellular phytases of *E. burtonii*; 0.15 ml 8 mM sodium phytate previously adjusted to pH as mentioned above with 1 N HCl; 0.20 ml enzyme preparation and 0.65 ml double distilled water. The final concentration of phytate was 1.0 mM. The reaction mixture was incubated for 30 min at temperature as mentioned above. After incubation, samples were withdrawn, deproteinized by adding 0.80 ml 10% TCA and total protein was analyzed by Lowry method (Lowry *et al.*, 1951). Further procedure and deter-mination of phytase activity by following the procedure developed by Sutardi and Buckle (1988).

Effect of pH

The effect of pH on phytase activity was determined in 0.1 M acetate buffer with final concentration 1.0 mM sodium phytate as substrate, incubated for 30 min at temperatures specified above. Mixtures were adjusted to pH 3.2 – 4.3 and 2.4 – 4.1 at 0.1 pH intervals for extra- and intracellular phytases of *E. burtonii*, and to pH 3.6 – 4.7 and 4.4 – 5.5 at 0.1 pH intervals for extra- and intracellular phytases of *C. diddensiae* and *C. tropicalis*, respectively.

Effect of temperature

Phytase assay mixtures were incubated for 30 min over the temperature range 35° – 70°C at 5 degree C intervals and 70° – 90°C at 10 degree C intervals.

Effect of substrate concentration

Optimum substrate concentration for phytase activity was determined by following the procedure as described by Sutardi and Buckle (1988) except that sodium phytate concentration were from 0 – 0.25 mM at 0.05 mM intervals and from 0.25 – 2.00 mM at 0.25 mM intervals. K_m and V_{max} values were estimated by the method of Lineweaver and Burk (1934).

Rate of denaturation

Partially purified enzyme was heated at 70° and 80°C, 60° and 75°C, and 55° and 65°C for extracellular phytases of *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively, and at 65° and 75°C, 55° and 65°C, and 45° and 55°C for intracellular phytases of *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively, for 0 – 60 min at intervals of 10 min, cooled, sodium phytate added to a final concentration of 1.0 mM and phytases assayed by using the procedure modified by Sutardi and Buckle (1988). The energy of inactivation and temperature coefficient were estimated by the Arrhenius equation.

Thermal inactivation for phytase

Enzymes in buffer solution were heated in a waterbath for 10 min over the temperature range 40° – 100°C, cooled, sodium phytate added to a final concentration of 1.0 mM and phytases were assayed by following the procedure developed by Sutardi and Buckle (1988).

Effect of incubation time and temperature

Phytase activity was assayed during incubation of reaction mixtures at 40°, 50°, 60°, 70°, and 80°C for extracellular phytases of *E. burtonii* and *C. diddensiae*, at 30°, 40°, 45°, 50°, and 60°C for extracellular phytase of *C. tropicalis*, at 40°, 50°, 55°, 60°, 65°, and 70°C for intracellular phytases of *E. burtonii* and *C. diddensiae*, and at 30°, 40°, 45°, 45°, 50°, and 55°C for intracellular phytase of *C. tropicalis* for times from 30 to 180 min at 30 min intervals. The energy of activation for phytase was estimated from the Arrhenius equation.

RESULTS AND DISCUSSION

Production of phytase

E. burtonii, *C. diddensiae* and *C. tropicalis* grown in Malt Extract Broth (MEB) medium produced both extra- and intracellular phytases. Relatively little is known about the production of yeast phytases; in one survey of a range of microorganisms for production of extracellular phytase, none of the yeasts examined was found to produce phytase (Shieh and Ware, 1968). Nayini and Markakis (1984) reported that baker's yeast produced an intracellular phytase, but extracellular phytase was not described.

In the present study the partially purified yeast extracellular phytases showed higher total activity than did the intracellular phytases (Table 1). *E. burtonii* produced significantly higher levels of extra- and intracellular phytases, while *C. diddensiae* produced the lowest levels of extra- and intracellular phytases. Wang *et al.* (1980) reported that some strain of *A. oryzae* grown in synthetic medium produced higher extracellular phytase activity than intracellular phytase. Some of them produced the same level of enzymes when they were grown in rice medium.

Table 1. Yield and specific activities during purification of extra- and intracellular yeast phytases of *E. burtoniii, C. diddensiae* and *C. tropicalis*.

Purification Stage	Yeast	Extracellular phytase						Intracellular phytase					
		Total Vol. (ml)	Total Protein (mg)	Total Activity (unit)*	Specific Activity (unit/mg)	Recovery (%)	Purification (fold)	Total Vol. (ml)	Total Protein (mg)	Total Activity (unit)*	Specific Activity (unit/mg)	Recovery (%)	Purification (fold)
Culture Filtrate	*E. burtonii*	1725	1729	7.3	0.004	100	-	94	468	6.9	0.015	100	-
	C. diddensiae	1725	1776	1.0	0.001	100	-	86	339	0.3	0.001	100	-
	C. tropicalis	1725	1754	4.7	0.003	100	-	95	480	0.8	0.002	100	-
Freeze drying And dialysis	*E. burtonii*	195	421	5.9	0.014	81	3.5	90	121	5.9	0.049	86	3.3
	C. diddensiae	196	491	0.4	0.001	40	1.5	84	160	0.2	0.002	67	2.0
	C. tropicalis	178	406	2.4	0.006	51	2.0	94	154	0.3	0.002	38	1.0
Aceton Fractionation, Dialysis and Freeze drying	*E. burtonii*	36	17.4	5.3	0.303	73	76	40	2.7	2.8	1.040	41	69
	C. diddensiae	34	10.0	0.3	0.030	30	30	36	5.6	0.2	0.036	67	36
	C. tropicalis	38	22.3	1.0	0.045	22	15	41	2.0	0.2	0.100	25	50

* One unit of enzyme is that amount of protein that can liberate 1 mole P_i per min from the respective substrate under experimental conditions. Phytase was assayed by using sodium phytate (1 mM) as substrate.

Isolation and purification of phytases

Both extra- and intracellular phytases were isolated from growth media and yeast cells, respectively. Yield and specific activities of the phytases at various stages of purification are presented in Table 1. At each stage of purification, specific activities of the intracellular phytases were higher than those of the extracellular enzyme except in the culture filtrates (before purification), and after freeze drying and dialysis of phytases from *C. tropicalis*.

The extracellular phytases of *E. burtonii*, *C. diddensiae* and *C. tropicalis* were purified 72, 46 and 17 fold, respectively, with recoveries 72.6, 26.3 and 21.9%, respectively. The intracellular phytases were purified by 71, 33 and 59 fold, with recoveries of 40.9, 56.7 and 25.3% for *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively. The purification achieved by acetone fractionation was substantially higher than that obtained by freeze drying and dialysis.

Effect of pH

The effect of pH on both extra- and intracellular yeast phytases is shown in Figure 1. The pH optima of extra- and intracellular phytases from *C. diddensiae* and *C. tropicalis* were 4.2 and 4.8, respectively, while pH optima of extra- and intracellular phytases from *E. burtonii* were 3.9 and 3.2, respectively. The pH optima for all species of yeasts examined characterize the enzymes as acid phosphohydrolases as reported by Wang *et al.* (1980) for phytase from *A. oryzae*. Various pH optima have been reported by a number of investigators for the cellular phytase from microorganisms (Sutardi, 1988). In the present study, extracellular phytase activities increased moderately before and after the optimum pH. The extracellular phytase activity of *C. tropicalis* was destroyed above pH 5.3 with about 75% loss of activity.

Intracellular phytase activity of *E. burtonii* increased slightly before the optimum pH but diminished moderately at pHs above the optimum pH, while intracellular phytase activity of *C. diddensiae* increased sharply before and after the optimum pH. More than 70 and 90% loss of activity was recorded when the pH decreased to below 3.7 and rose to above 4.4, respectively.

Intracellular phytase activity of *C. tropicalis* increased moderately up to pH 4.7 and sharply up to the pH optimum; above pH 4.9 the enzyme activity decreased moderately. Above pH 5.3 more than 80% loss of activity occurred. The pH optima of the three yeast phytases are within the range (pH 2.2 – 5.6) reported for microbial phytases (Graf, 1986).

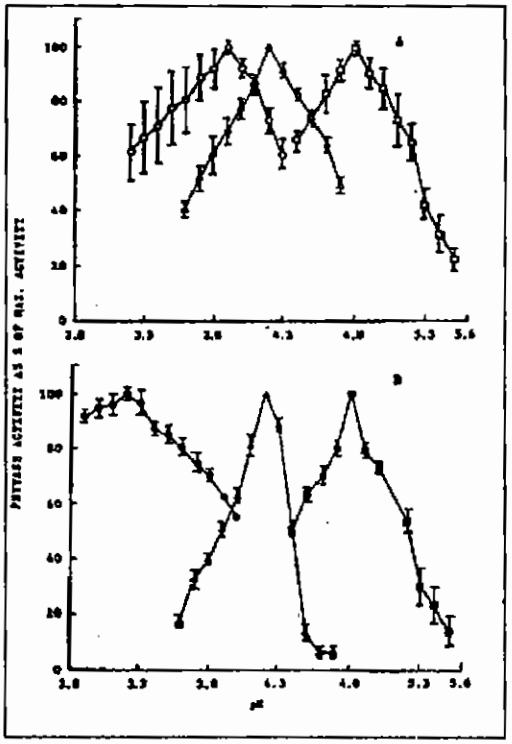

Figure 1. The pH activity profile of extra – (A) and intracellular (B) phytases from *E. burtonii* (m,l), *C. diddensiae* (△,s) and *C. tropicalis* (□,n). Vertical bars indicate range of duplicate determination on each of 2 enzyme preparations.

Effect of temperature

The temperature-activity profile of the extracellular phytases from *E. burtonii*, *C. diddensiae* and *C. tropicalis* show maximum activity at 70°, 60°, and 55°C, respectively, while intracellular phytases had maximum activity at 65°, 55° and 45°C, respectively (Figure 2). Inactivation of the extracellular phytases commenced above 80°C for *E. burtonii* and *C. diddensiae*, and above 65°C for *C. tropicalis*. Incubation above these temperatures caused losses of activity of over 90%, and at 100°C the enzymes were completely destroyed.

As the temperature increased from 37°C to the optimum temperature, extracellular phytase activities increased about 5 folds, while intracellular phytase activities increased about 5, 10 and 2.5 folds for *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively. Inactivation of the intracellular phytases commenced above 70°C for *E. burtonii* and *C. diddensiae*; at 80°C the activities were completely destroyed. The intracellular phytase of *C. tropicalis* was inactivated at above 60°C, and at 100°C caused 90% loss of activity. The temperature optima found for the yeast phytases were within the range (40° – 70°C) reported by previous investigators (Sutardi, 1988).

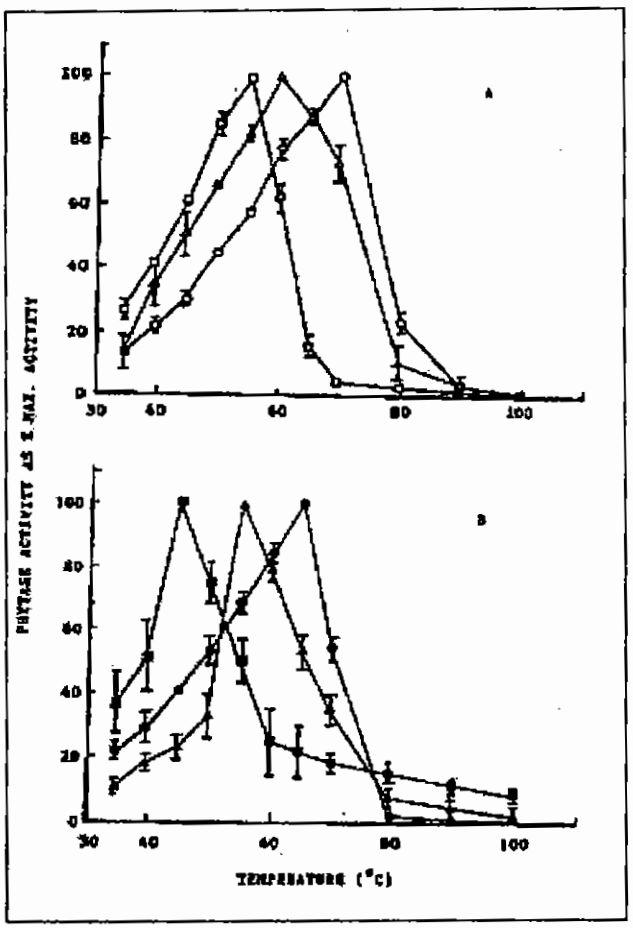

Figure 2. The temperature-activity profile of extra- (A) and intracellular (B) phytases from *E. burtonii* (m,l), *C. diddensiae* (Δ,s) and *C. tropicalis* (□,n).Vertical bars indicate range of duplicate determinations on each of 2 enzyme preparartions.

Effect of substrate concentration

The effect of substrate concentration on phytase activity is illustrated in Figure 3. Similar to phytase from various sources, the activites of extra- and intracellular yeast phytases were inhibited by high concentrations of phytic acid. Extracellular phytases from *E. burtonii* and *C. diddensiae* were inhibited by concentrations of phytic acid greater than 1.0 mM, while the extracellular phytase from *C. tropicalis* was inhibited by a phytic acid concentration greater than 1.75 mM. Like the extracellular phytases, the intracellular phytases were inhibited also at phytic acid concentrations greater 1.0 mM.

Both extra- and intracellular phytases of *C. diddensiae* had maximum activity at 1.0 mM substrate concentration under optimum assay conditions, abobe which the activity marginally decreased.

The initial reaction velocity was proportional at substrate concentration up to 1.0 mM. However, above 1.0 mM, activity increased less rapidly and the rate of increase in activity was no longer proportional to the substrate concentration. Except for the phytases from *C. diddensiae*, typical rectangular hyperbole curves were obtained, following the Michaelis-Menten's equation.

Nayini and Markakis (1984) reported that baker's yeast was inhibited at a phytic acid concentration of 1.0 mM and at 10 mM the activity was completely terminated. Significant inhibition at 0.40 – 0.75 mM phytic acid concentration was observed for mould phytase of *Rhizopus oligosporus* (Sutardi and Buckle, 1988).

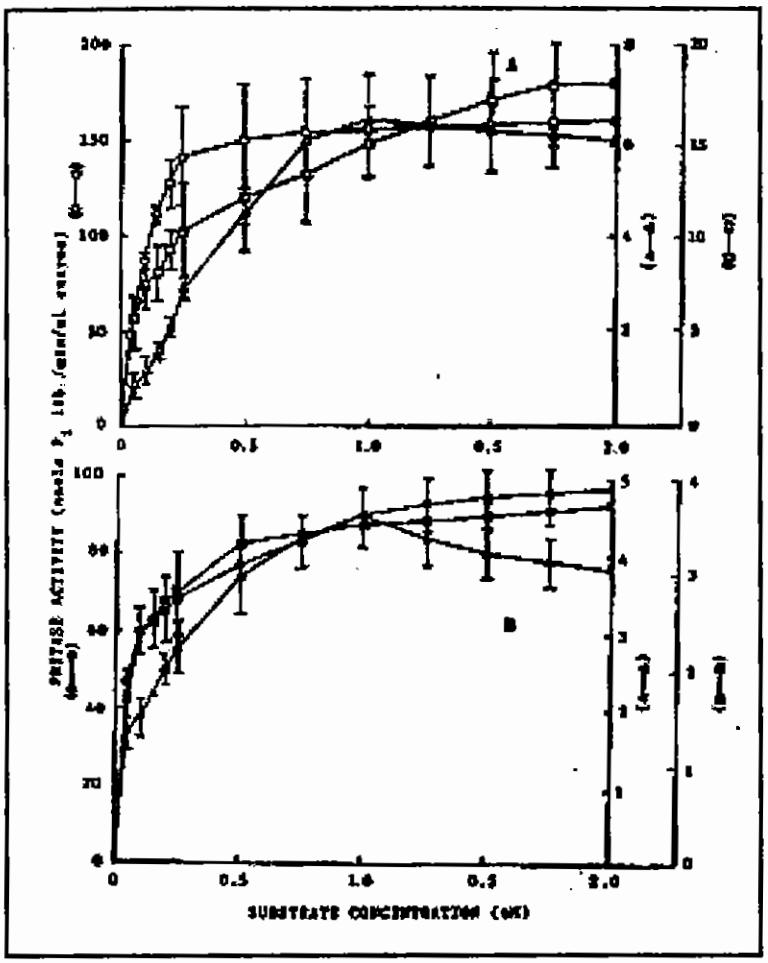

Figure 3. Effect of substrate concentration on extra- (A) and intracellular (B) phytases from *E. burtonii* (m,l), *C. diddensiae* (Δ,s) and *C. tropicalis* ([],n).Vertical bars indicate range of duplicate determinations on each of 2 enzyme preparartions.

Based on the results shown in Figure 3, a double reciprocal plot of substrate concentration against reaction rate produced K_m values of 1.4 x 10^{-4}, 3.1 x 10^{-4} and 1.0 x 10^{-4}M for extracellular phytases from *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively. For the intracellular phytases from *E. burtonii*, *C. diddensiae* and *C. tropicalis*, K_m values were 0.5 x 10^{-4}, 0.7 x 10^{-4} and 0.6 x 10^{-4}M, respectively (Figure 4). The K_m values for extra- and intracellular phytases were within the range (10^{-2} – 10^{-6}M) reported by Palmer (1981). The K_m values of extracellular phytases were relatively higher compared with the K_m values for the intracellular phytases, indicating weak affinity between the former enzymes and the substrate.

V_{max} values, determined by the same manner as for the K_m values, were 0.19, 0.05 and 0.02 mole P_i liberated/min/ml enzyme for extracellular phytases from *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively; and were 0.09, 0.004 and 0.004 mole P_i liberated/min/ml enzyme, respectively for intracellular phytases of *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively. Extracellular yeast phytases had higher V_{max} values than did intracellular yeast phytases.

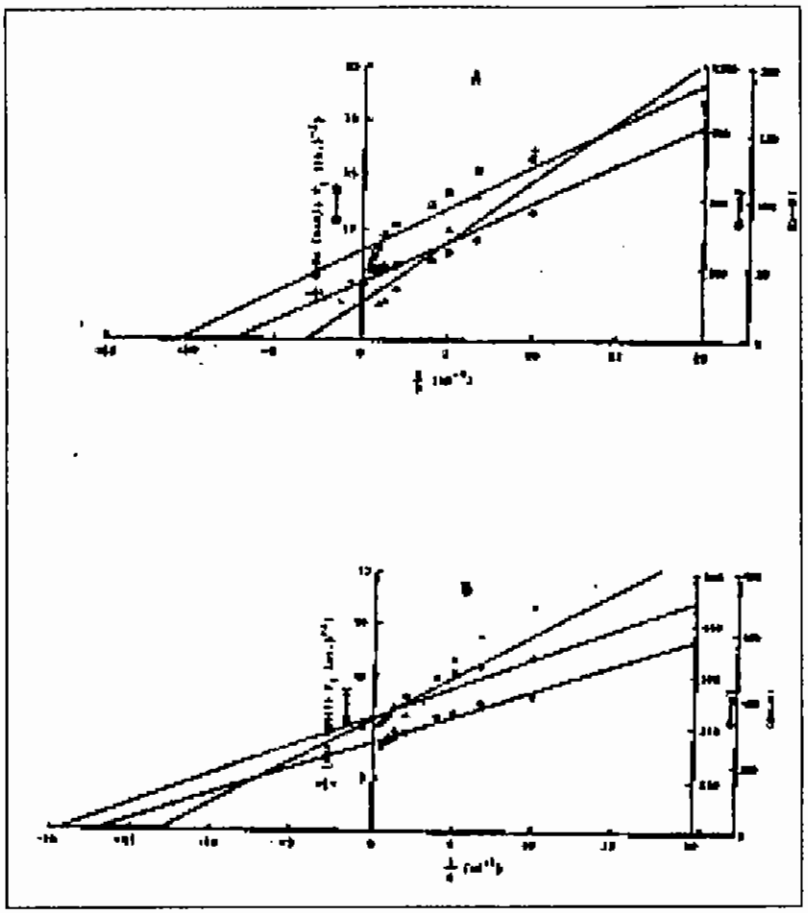

Figure 4. Lineweaver-Burk plot for activity of extra- (A) and intracellular (B) phytases from *E. burtonii*, (m,l), *C. diddensiae* (Δ,s) and *C. tropicalis* (□,n). Data obtained from. Figure 3 are ploted as reciprocal of the initial rate (1/v, min.μmole^{-1}) versus reciprocal of the substrate concentration (1/s, mM^{-1}).

V_{max} of extra- and intracellular phytases from *E. burtonii* were higher compared to those V_{max} of extra- and intracellular phytases from *C. diddensiae* and *C. tropicalis*. Comparison with the V_{max} of other microbial phytases cannot be made because of the differences in the units used (Sutardi, 1988). In the present study V_{max} of yeast phytases is lower than the V_{max} of mould phytase (0.076 – 0.34 μmol P_i per min per ml enzyme) (Sutardi and Buckle, 1988).

Rate of denaturation

Extra- and intracellular phytase activities of *E. burtonii*, *C. diddensiae* and *C. tropicalis* remaining after heating at temperatures as specified are shown in Figure 5. The extracellular phytase of *E. burtonii* shows a large difference in rate of denaturation at 70°C and 80°C. Heating this phytase at 80°C for 10 min reduced phytase activity to very low levels compared to the another two extracellular phytases. Incubation at temperature above the optimum temperature increased the rate of denaturation of the enzymes, as did prolonged heating.

The inactivation-time relationship deviated significantly from linearity, and two inactivation rate constants (k) can be estimated by the procedure described above. Extracellular phytases from *E. burtonii*, *C. diddensiae* and *C. tropicalis* had inactivation energy of 76.800, 70,500 and 18,600 cal/mole, respectively. The temperature coefficient of inactivation (Q_{10}) over the intervals of 70° – 80°C, 60° – 70°C, and 55° – 65°C for extracelllar phytases of *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively, were 25, 25 and 2. Inactivation energies and Q_{10} of the extracellular phytases were relatively high compared with those for various extracellular phytases reported by previous investigators (Sutardi, 1988).

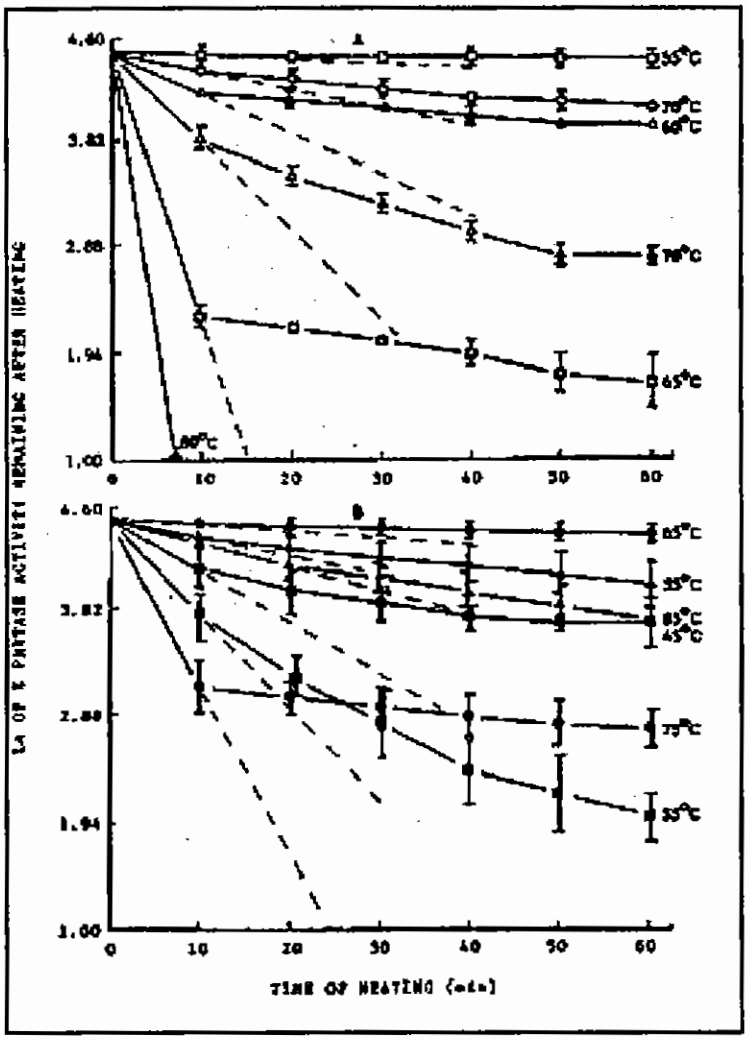

Figure 5. Rate of denaturation of extra- (A) and intracellular (B) phytases from *E. burtonii* (m,l), *C. diddensiae* (△,s) and *C. tropicalis* (□,n). Vertical bars indicate range of duplicate determination on each of 2 enzyme preparations.

Intracellular phytases from *E. burtonii*, *C. diddensiae* and *C. tropicalis* had inactivation energies of 6,300, 12,600 and 14,900 cal/mole, respectively. The temperature coefficients of inactivation were 15, 2 and 2, respectively. The relatively high values in activation energies and Q_{10} of the extracelluar phytases from *E. burtonii* and *C. diddensiae* cannot be explained.

Thermal inactivation

Figure 6 shows the effect of heating for 10 min on thermal inactivation of yeast phytases. Incubation of the heated enzyme preparation for 10 min at up to 60°C marginally depressed extracellular phtase activity of *E. burtonii*, but increased significantly the activity of extracellular phytase from *C. diddensiae*; the extracellular phytase of *C. tropicalis* increased in activity at 50°C, but decreased at temperatures of 60°C and above.

All extracellular phytase activities decreased sharply at temperatures above 60°C, while at temperature above 80°C all enzyme activities were practically absent. Heating of the extracellular phytase from *C. diddensiae* at temperature below 60°C or below its optimum temperature (60°C) appear to activate rather than inactivate the phytase. A similar activation effect occurred when the extracellular phytase of *C. tropicalis* was heated for 10 min at 50°C (below its optimum temperature, 55°C), but not for extracellular phytase of *E. burtonii* which has a higher optimum temperature (i.e. 70°C). The intracellular phytase of *E. burtonii* was highly activated on heating for 10 min at temperature up to 70°C indicating thermostability of the enzyme. In contrast, activities of intracellular phytases from *C. diddensiae* and *C. tropicalis* were significantly decreased at temperature as low as 40°C. At temperature at or above 80°C intracellular phytase activities were almost completely destroyed, and no activity remained for the intracellular phytase of *E. burtonii*.

Thus heating at 80°C or above completely destroyed both extra- and intracellular yeast phytases, in agreement with the observation by Yamada *et al.* (1968) that the phytase from *Aspergillus terreus* was effectively destroyed at 80°C.

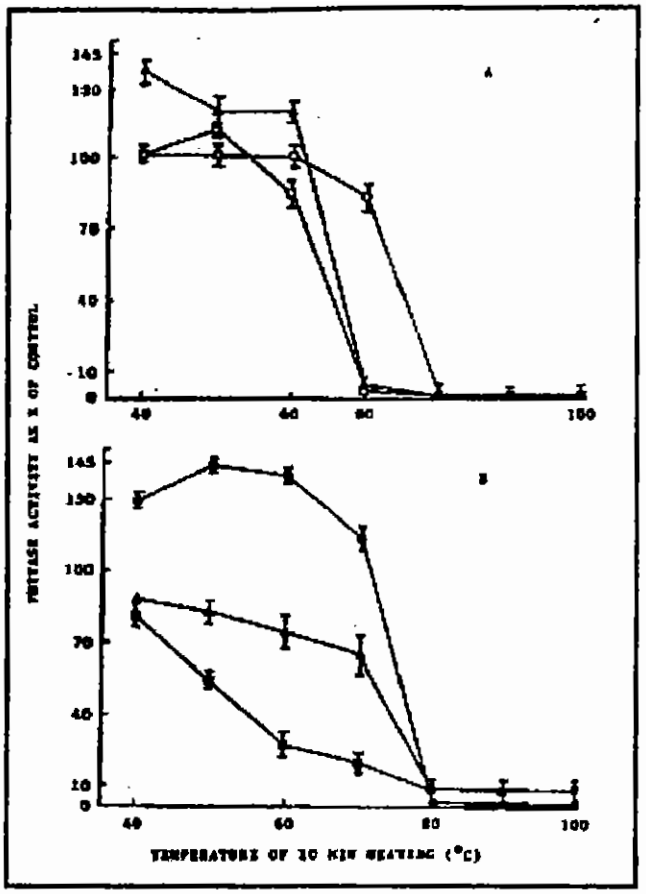

Figure 6. Thermal inactivation of extra- (A) and Intracellular (B) phytases from *E. burtonii* (m,l), *C. diddensiae* (Δ,s) and *C. tropicalis* ([],n). Vertical bars indicate range of duplicate determination on each of 2 enzyme preparation.

Effect of incubation time and temperature

Table 2, 3 and 4 show the effects of incubation time and temperature on phytic acid hydrolysis by extra- and intracellular phytases from *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively. Increasing levels of P_i were liberated from phytic acid with increase in temperature up to the temperature optima for 180 min incubation; P_i decreased sharply on further rise of temperature. Extra- and intracellular phytases of *E. burtonii* at 80° and 70°C had only about 3.5 and 7.0% of the activities compared to those at 70° and 65°C, respectively after 180 min incubation (Table 2). Incubation of extra- and intracellular phytases from *E. burtonii* for various times (30 – 180 min) at 80°C showed very low activity but increasing activity, while for extracellular phytase of *C. diddensiae* the activity did not change. It is probably due to the denaturation of the enzyme. At 70° and 65°C the activities of extra- and intracellular phytases from *C. diddensiae* decreased about 50 and 28% of the activities compared to those at 60° and 55°C, respectively (Table 3). At 80°C both extra- and intracellular phytases from *C. diddensiae* has activity less than 15% after 180 min incubation, while extra- and intracellular phytases of *C. tropicalis* at 60° and 50°C had about 17.0 and 30.0% of the activities of those at 55° and 45°C, respectively after 180 min incubation (Table 4).

Table 2. Effects of incubation time and temperature on the liberation of P_i from phytate by extra- and intracellular phytases from *E. burtonii*.

Temperature (°C)	Extracellular phytase activity at incubation time (min)*						Intracellular phytase activity at incubation time (min)*					
	30	60	90	120	150	180	30	60	90	120	150	180
40	0.8± 0.02	1.4± 0.01	2.0± 0.13	2.5± 0 10	3.1±0.06	3.6± 0 00	4.4± 0.55	7 8±0 97	11.3±0 20	13 6±0 28	15.7±0 31	17.4±0 86
50	1.1± 0.00	2.1± 0.03	3.0± 0 25	3 8± 0 31	4 7±0.14	5 4± 0 28	6 4± 0.45	10 0±0.25	13 8±0 06	16 5±0 25	18 2±0 91	18 5± 0 39
55	-	-	-	-	-	-	6 9± 0 85	12 9±0 08	18 1±0 13	20 1±0 90	21 5±0 03	22.5± 0 05
60	1 9± 0 05	3 2± 0 27	4 4± 0 21	5 2± 0 21	6 0±0 13	6 7± 0 17	7 3± 0 98	13.9±0 02	18 8±0 37	21 0±0 38	21.9±0.34	23 0± 0 57
65	-	-	-	-	-	-	7 9± 0 10	14.1±0 41	19 0±0 34	21 7±0 70	22.8±0.73	23.6± 0 57
70	2.3±0.14	4 1± 0.36	5 6± 0 39	6 7±0 40	7 9±0.54	8.7±0 70	1 5± 0 01	1 7±0 06	1 7±0 03	1 7±0.01	1.7±0 01	1.7± 0 02
80	0.2± 0.06	0.3± 0.09	0.3± 0.11	0.3±0.10	0.3±0.05	0.3±0 04	-	-	-	-	-	-

* Activity defined as µmole P_i liberated per ml acetone purified enzyme.

\# Mean ± s.d. of duplicate determinations on each of two enzyme preparations.

Table 3. Effects of incubation time and temperature on the liberation of Pi from phytate by extra- and intracellular phytases from *C.diddensiae*.

Temperature (°C)	Extracellular phytase activity at incubation time (min)*						Intracellular phytase activity at incubation time (min)*					
	30	60	90	120	150	180	30	60	90	120	150	180
40	0.05±0.00	0.10±0.01	0.14±0.02	0.18±0.00	0.22±0.01	0.25±0.01	0.12±0.05	0.20±0.06	0.27±0.06	0.34±0.02	0.40±0.02	0.44±0.05#
50	0.10±0.00	0.20±0.01	0.29±0.02	0.34±0.01	0.37±0.04	0.39±0.04	0.17±0.01	0.31±0.02	0.41±0.01	0.51±0.03	0.57±0.01	0.60±0.00
55	-	-	-	-	-	-	0.21±0.08	0.37±0.03	0.51±0.09	0.63±0.02	0.74±0.01	0.80±0.01
60	0.15±0.03	0.25±0.07	0.35±0.03	0.39±0.02	0.41±0.01	0.42±0.00	0.10±0.04	0.18±0.02	0.25±0.07	0.30±0.04	0.34±0.07	0.36±0.02
65	-	-	-	-	-	-	0.04±0.01	0.08±0.00	0.13±0.01	0.17±0.03	0.20±0.00	0.22±0.02
70	0.10±0.05	0.17±0.01	0.19±0.01	0.21±0.00	0.22±0.01	0.22±0.03	0.00±0.00	0.02±0.00	0.03±0.01	0.05±0.01	0.06±0.02	0.07±0.01
80	0.03±0.01	0.04±0.00	0.05±0.00	0.06±0.01	0.06±0.00	0.06±0.00	0.00±0.00	0.02±0.00	0.03±0.01	0.05±0.02	0.06±0.01	0.07±0.02

* Activity defined as µmole P_i liberated per ml acetone purified enzyme.

\# Mean ± s.d. of duplicate determinations on each of two enzyme preparations.

Table 4. Effects of incubation time and temperature on the liberation of P_i from phytate by extra- and intracellular phytases from *C. tropicalis*.

Temperature (°C)	Extracellular phytase activity at incubation time (min)*						Intracellular phytase activity at incubation time (min)*					
	30	60	90	120	150	180	30	60	90	120	150	180
30	0.23±0.01	0.35±0.01	0.49±0.01	0.60±0.01	0.73±0.02	0.85±0.00	0.05±0.00	0.10±0.00	0.17±0.00	0.22±0.03	0.25±0.05	0.27±0.02#
40	0.34±0.00	0.61±0.01	0.91±0.00	1.12±0.00	1.33±0.01	1.35±0.03	0.17±0.00	0.26±0.03	0.33±0.02	0.36±0.01	0.37±0.01	0.38±0.06
45	0.47±0.01	0.87±0.00	1.31±0.03	1.63±0.05	1.95±0.04	2.10±0.03	0.24±0.03	0.36±0.04	0.40±0.03	0.44±0.05	0.47±0.01	0.49±0.02
50	0.66±0.02	1.23±0.02	1.78±0.06	2.19±0.05	2.60±0.05	2.90±0.03	0.00±0.00	0.00±0.00	0.10±0.00	0.12±0.01	0.14±0.00	0.15±0.02
55	0.76±0.00	1.39±0.02	1.94±0.05	2.35±0.03	2.78±0.01	3.12±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.00	0.06±0.00	0.08±0.01
60	0.19±0.00	0.40±0.03	0.40±0.03	0.47±0.01	0.50±0.01	0.52±0.01	-	-	-	-	-	-

* Activity defined as µmole P_i liberated per ml acetone purified enzyme.

\# Mean ± s.d. of duplicate determinations on each of two enzyme preparations.

The highest activities were observed at 70°, 60° and 55°C for extracellular phytases of *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively, and at 65°, 55° and 45°C for the intracellular phytases from *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively, which are consistent with previous observation in the present study (Figure 2).

From the data in Table 2, 3 and 4, the activation energies for the hydrolysis of phytic acid by extra- and intracellular phytases were calculated by the Arrhenius equation and the results are presented in Figure 7. Extracellular phytases of *E. burtonii*, *C. diddensiae* and *C. tropicalis* had activation energies of 7,100; 9,100 and 13,000 cal/mole, respectively; while the intracellular phytases of these organisms had activation energies of 5,600; 7,100 and 21,700 cal/mole, respectively.

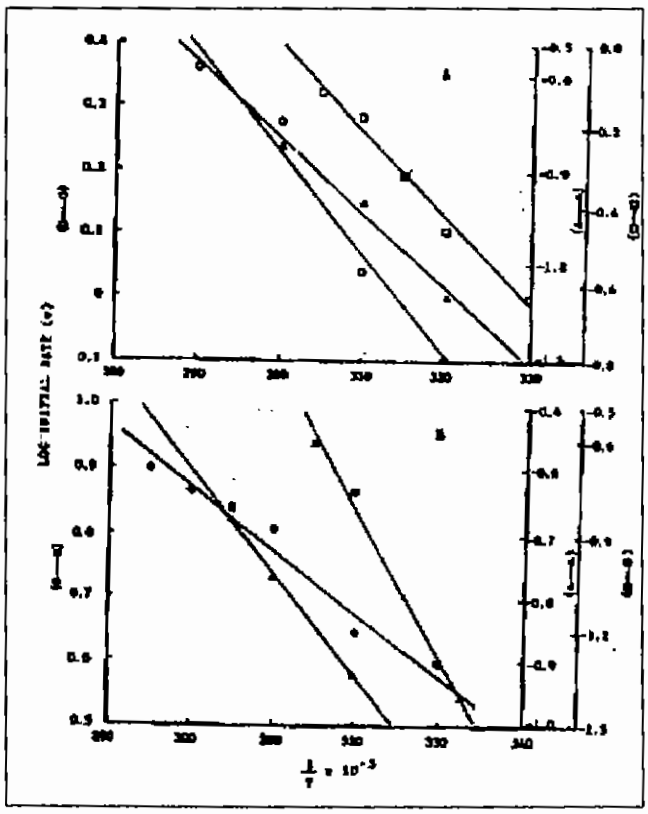

Figure 7. Arrhenius plots for estimation of activation energy from the hydrolysis of phytic acid by extra- (A) and intracellular (B) phytases from *E. burtonii* (m,l), *C. diddensiae* (Δ,s) and *C. tropicalis* (□,n). Initial rate (v) estimated from data of Table 2, 3 and 4; T = absolute temperature.

CONCLUSSION

E. burtonii, *C. diddensiae* and *C. tropicalis* grown in MEB medium produced both extra- and intracellular phytases. The extracellular phytases of *E. burtonii*, *C. diddensiae* and *C. tropicalis* were purified 72, 46 and 17 fold, respectively with recoveries 72.6, 26.3 and 21.9%, respectively. The intracellular phytases were purified by 71, 33 and 59 fold, with recoveries of 40.9, 56.7 and 25.3% for *E. burtonii*, *C. diddensiae* and *C. tropicalis*, respectively.

The optimum pH of extra- and intracellular phytases from *C. diddensiae* and *C. tropicalis* were 4.2 and 4.8, respectively, while pH optimum of extra- and intracellular phytases from *E. burtonii* were 3.9 and 3.2, respectively. The temperature-activity profile of the extra- and intracellular phytases were at 45° – 70°C for *E. burtonii*, *C. diddensiae* and *C. tropicalis*.

Estimated K_m values for extra- and intracellular phytases from yeast were within the range $0.5 - 3.1 \times 10^{-4}$ M. The V_{max} values were within the range 0.004 – 0.19 µmole P_i liberated/min/ml enzyme for the extra- and intracellular phytases of *E. burtonii, C. diddensiae and C. tropicalis*.

Extracellular phytases of *E. burtonii*, *C. diddensiae* and *C. tropicalis* had activation energies of 7,100, 9,100 and 13,000 cal/mole, respectively; while the intracellular phytases of these organisms had activation energies of 5,600, 7,100, and 21,700 cal/mole, respectively.

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